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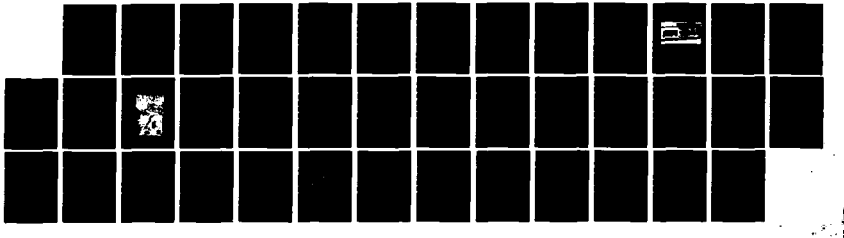

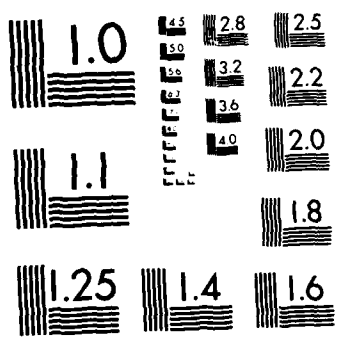

FINAL TECHNICAL REPORT

(NO. 6)

CONTRACT NUMBER N00014-79-C0332

"Studies of Electrically Stimulated Rat Limb and Peripheral Nerve Regeneration"

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40536-0084

STUDIES OF ELECTRICALLY STIMULATED RAT LIMB AND PERIPHERAL NERVE REGENERATION

The studies carried on under this contract were essentially divided both physically and scientifically into two areas, as outlined in the title. The studies of rat limb regeneration were carried out by the Principal Investigator at the University of Kentucky, while the studies of peripheral nerve regeneration were carried out by Dr. William G. Winter at the University of Colorado and the Denver V.A. Hospital. Accordingly, this technical report is divided into two sections; one prepared by the Principal Investigator, the other by Dr. Winter. If questions arise in the mind of the reader, they should perhaps be directed to the individual responsible for the work. The P.I.'s address is on the facing page of the report. Dr. Winter may be reached at the Department of Orthopedic Surgery, V.A. Medical Center, 1055 Clermont St., Denver, CO 80220.

I. Studies of Electrically Stimulated Rat Limb Regeneration

A. Background:

Earlier studies by the principal investigator (1) had demonstrated that induction of limb regeneration was possible in amphibians by the use of very small (ca. 10 - 100 microamperes per mm^2) direct electrical currents. Accordingly, an attempt was made under the terms of this contract to produce similar regeneration in a mammal, the rat being chosen on grounds of convenient size, cost, and ease of manipulation.

The experiments described here fall into three more or less discrete groups; stimulation with direct current, with

magnetically induced current, and with a combination of both. Therefore, a description of the experiments will also be arranged in tripartite form to correspond to the flow of the experimental design.

B. Experiments:

1. Direct Current:

The results of these experiments have been published previously, and a reprint is attached. To produce the results obtained, we began by amputating the right forelimbs of young male and female Sprague-Dawley rats. The animals were obtained at an age of 4 weeks, and were then held in the laboratory for an additional four weeks to ensure their good health and normal development. As they reached 8 weeks, they were moved into the experiments by amputing the right forelimb at the midpoint of the zygopodium. Amputation at this point lay below most of the major muscle masses, and bleeding was minimal, so that no attempts were made to close the wound or apply hemostasis. This consideration is important, since it has been shown for amphibians that surgical closure of the wound which brings the dermis over the amputation surface is absolutely inhibitory to regeneration. Accordingly, we left the wounded surfaces open and exposed. With careful cage maintenance and frequent cleaning, we experienced absolutely no difficulties with infection or with excessive bleeding. Bleeding of the stump invariably stopped within 60 seconds, and was never excessive. Such was generally the case if the amputations were done with scissors rather than a scalpel. The crushing action of the scissors apparently released vasoconstrictive agents which

promptly inhibited extravasation. Amputations with a scapel were avoided, since they did bleed profusely, and required cauterization.

Following amputation, a second wound was made in the midline of the back between the shoulders. A pocket was formed there by blunt dissection, and a battery-resistor circuit of the type seen in Fig. 1 was implanted. The circuits were made with a Mallory RM 312 or equivalent mercury cell, delivering 1.35 V, and either a 1.3 or 13 megohm resistor. The lead wires were composed of pure platinum for the anode, and a 7-stranded TFE-insulated .007 in. diameter stainless steel for the cathode. Leads were attached with silver-filled epoxy to avoid heat damage to the batteries. The entire circuits, except for the leads were double-encapsulated; first in epoxy for physical stability, and then in Dow-Corning medical grade silicone rubber for immunocompatibility.

These circuits were implanted into the rats according to the diagram in Figure 2. Either the positive or negative lead was bent into the form of a zig-zag spring (a coil was tried, but did not work - the leads broke under flexion, and did not maintain contact with the wound surface). Then the lead was brought down the arm and placed in the dorsal-ulnar quarter of the limb stump. It was fastened in place by simply either spreading the strands of the stainless steel wire or making a tiny coil of the platinum in such a way that the zig-zag spring was brought into slight tension. This held the lead wire in contact with the wound surface, and also allowed for movements of the animals' forelimbs without disengaging the lead from contact, or producing undue

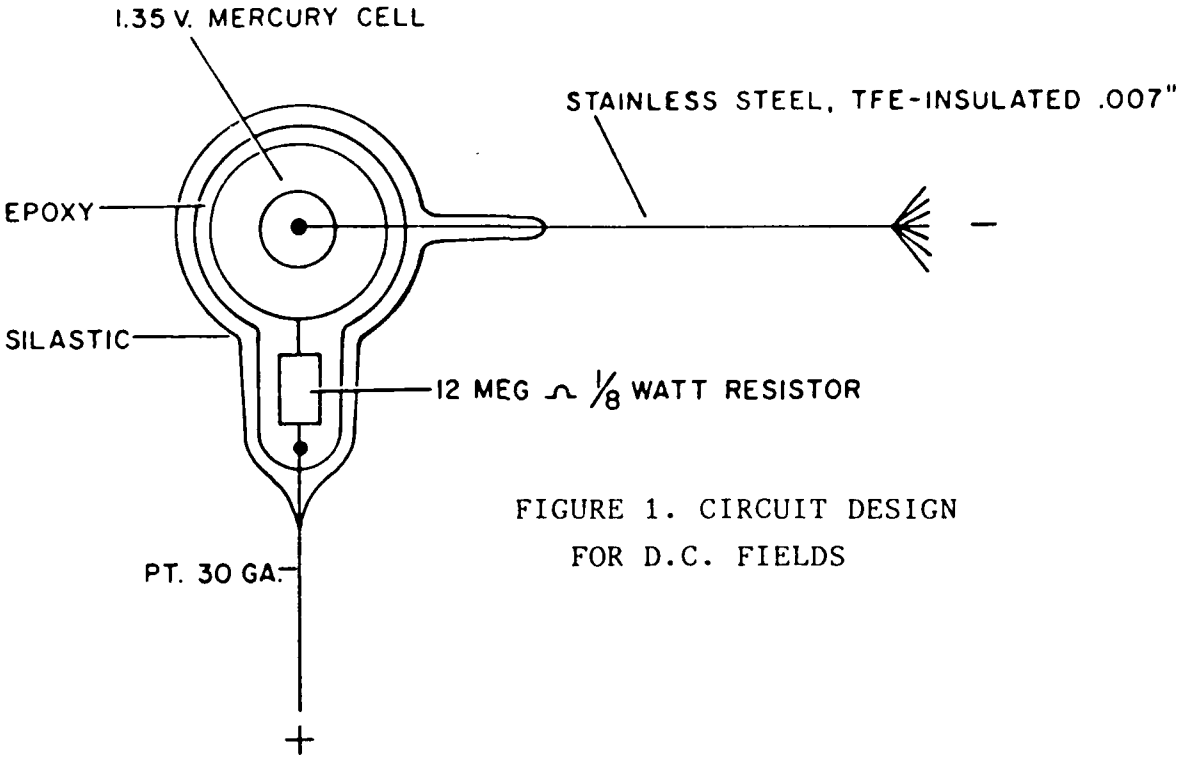


FIGURE 1. CIRCUIT DESIGN FOR D.C. FIELDS

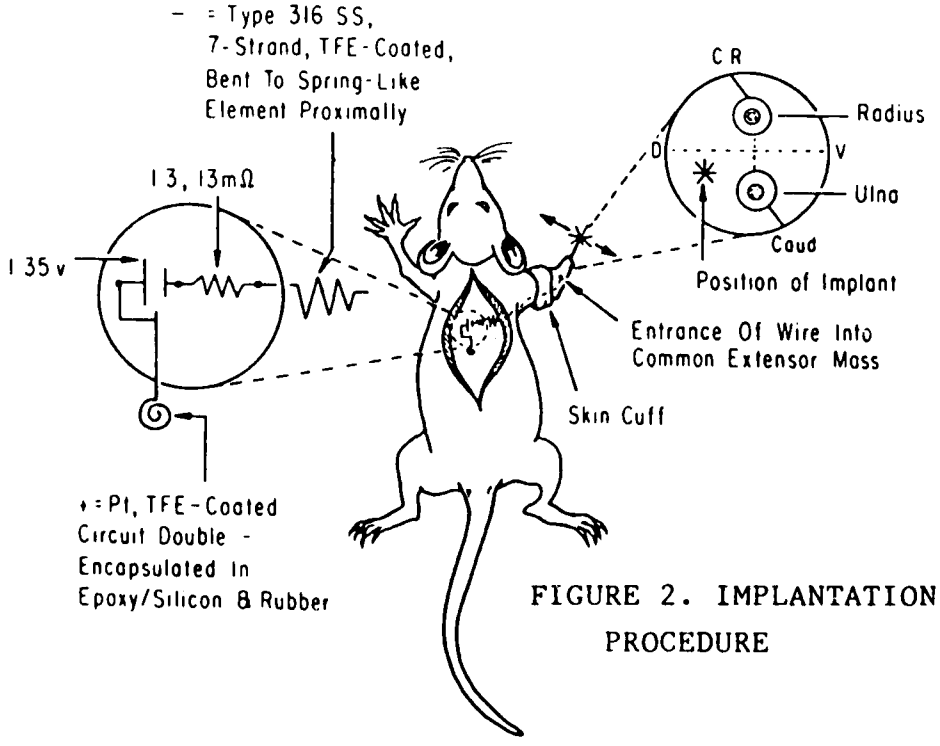


FIGURE 2. IMPLANTATION PROCEDURE

bending of the wires. Implantation of the wires into the dorsal-ulnar quarter of the stump was chosen as a result of earlier experiments with amphibians, which demonstrated the desirability of stimulating the stump in the position originally occupied by the apical ectodermal ridge during limb ontogenesis. Some trials were made with the electrodes in other positions, but the results were as with amphibians. The best regeneration was always obtained with the electrode placed in the dorso-ulnar quarter.

Table I illustrates the protocol followed, and Table II gives the results. No sexual dimorphism in the results appeared, so subsequent experiments were all performed on males, which were less expensive and generally more readily available.

To summarize, the direct current experiments clearly showed that limb regeneration in young rats is indeed possible. It is especially strongly induced when the negative lead was placed distally, and the current was set at approximately 1×10^{-6} Amperes per mm^2. As may be seen from the table, all 12 of the animals so treated produced cartilaginous outgrowth, and 10 of the 12 produced more than just cartilage, adding bone. Of even more interest, 8 of the 12 produced new structures which are characteristic of a regrowing limb - that is; new muscle tissue which attached appropriately to bone, and joints which were arranged in a proximal to distal sequence reminiscent of normal wrist and hand structures. Examination of the attached reprint will give some typical examples. Some trial experiments were also run at more elevated current levels, but they were invariably failures. If current levels were raised above those reported

TABLE I
PROTOCOL

Animals	Sex	Condition	Current density	Electrode at surface
3	M	Control	0	0
3	F	Control	0	0
6	M	Sham	0	3+, 3—
6	F	Sham	0	3+, 3—
12	M	Experimental	1×10^{-6} A/mm^2	6+, 6—
12	F	Experimental	1×10^{-6} A/mm^2	6+, 6—
12	M	Experimental	1×10^{-7} A/mm^2	6+, 6—
12	F	Experimental	1×10^{-7} A/mm^2	6+, 6—
Total 66				

TABLE II

RESULTS AFTER 60 DAYS

Condition	No.	Cartilage	Bone	Muscle	Joints	Outgrowth (mm)	*P* vs. *C* [a]
Control	6	6	0	0	0	0.10 ± 0.03	
Sham Op.	12	8	0	0	0	0.09 ± 0.055	N.S.
1.04×10^{-7} A/mm^2 Posit. out	12	12	1	0	0	0.46 ± 0.21	< 0.001
1.04×10^{-6} A/mm^2 posit. out	12	12	2	0	0	0.54 ± 0.21	< 0.001
1.04×10^{-7} A/mm^2 negat. out	12	12	6	5	3	1.64 ± 0.83	< 0.001
1.04×10^{-6} A/mm^2 negat. out	12	12	10	8	8	2.65 ± 1.08	< 0.001

[a] *P* = probability that the result is a chance occurrence; *C* = control.

here, the only results were formation of large amounts of disorganized connective tissue, indicating irritation, or actual tissue distruction if the current levels were as high as 1 milliampere per mm^2.

Unfortunately, none of the animals in these trials regenerated a complete new forelimb and hand, as did the frogs in our previous studies. However, the presence of well-organized distal elements, as well as the types of tissues formed, gave us some confidence that better results could perhaps be obtained under the right circumstances.

Detailed observations of the amputated limbs revealed that the amount of blastemal (formative) tissue which developed as a result of d.c. stimulation did not appear to be proportional to the amount produced in amphibian regeneration; it was much less in rats. Therefore, we made the assumption that some means should be tried to increase the amount of tissue formed, since it is obviously impossible to form a hand if there is insufficient tissue present to complete the regeneration. Accordingly, we attempted stimulation of amputated limb stumps by magnetically-induced electrical fields, as outlined in section 2.

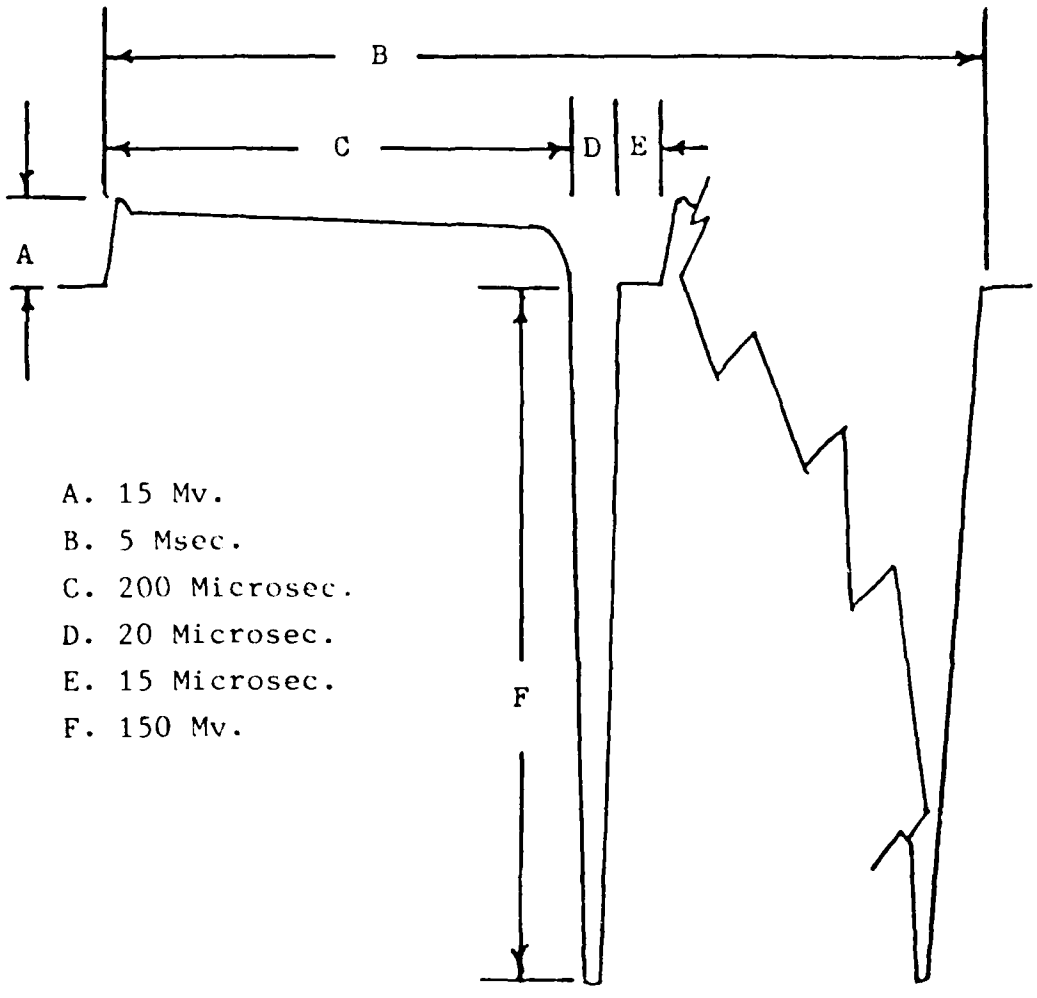
2. Magnetically-induced Pulsating Current:

Previous experiments on regenerating newt (salamander) limbs had demonstrated that magnetically induced pulsating electrical currents could speed regeneration by about 100-200%, and produce very large regenerates, indicating the formation of large amounts

of blastemal tissue. Accordingly, we decided to try using a similar method to increase the amount of tissue formed during blastema formation, hoping to thus induce more complete regeneration.

To accomplish this end, we used pulsed magnetic fields of the type becoming increasingly common for the treatment of non-united fractures in humans. As may be seen in Figure 3, the device used to produce the stimulation was essentially a pair of large nesting five-sided lucite boxes. The animal, an 8-week old male Sprague-Dawley rat, as described in section 1, had its right forelimb amputated. The animal was then placed in the inner box, whose open side faced upward. A second, larger lucite box was nested over the first, its open side thus facing the table surface. Access ports for water bottles and air exchange were arranged to match when the boxes were nested. The outer box was wrapped with four large equally spaced coils of #16 copper magnet wire (24 turns) attached in a parallel Helmholtz-aiding relationship to a specially modified Biosteogen pulse generator leased from Electro-Biology, Inc. As configured, the generator produced asymmetrical pulses in the coils. In essence, the fields consisted of trains of 27 pulses. Each train lasted 5 milliseconds, and the repetition rate of the trains could be varied in steps at 1,2,5,10,15, and 25 HZ. Individual pulses in the train took the form shown by Figure 4, as measured by a secondary coil composed of 65 turns of 36 Gauge (B&S) magnet wire with the leads shorted by a 10 Kohm resistor. This secondary coil is traceable to the National Bureau of Standards. The fields were

FIGURE 3. DEVICE USED TO STIMULATE WITH PULSED MAGNETIC FIELDS: Arrows point to coils surrounding the Lucite animal cages. A rat is visible inside the cage.



A. 15 Mv.
B. 5 Msec.
C. 200 Microsec.
D. 20 Microsec.
E. 15 Microsec.
F. 150 Mv.

FIGURE 4. PARAMETERS OF INDIVIDUAL PULSES

uniform within the boxes to within 10%. The major variation in the fields came at the very edge of the boxes, where the field strength dropped off very slightly within about 1 cm of the edge.

For sound theoretical reasons, it is impossible to deduce the exact strength of the fields in the cells and tissue at every point, but the fields in the bulk tissue fluids approach 1.5 mv and 1 microampere per cm^2. As previously stated, such fields have been shown to strongly influence regeneration of limbs in adult salamanders (2).

Because such large coils generate very extensive fields, it was necessary to keep the boxes at least 1.75 meters apart to avoid interference between the fields of adjacent coils. This large space requirement limited the number of animals that could be treated at once, since the amount of space (and the number of machines available) were not limitless. We were only able to treat 6 animals simultaneously, plus two concomitant controls in the center of the room. The protocol in Table III illustrates the experiments which were performed. The frequency survey was performed, since experiments in other systems had shown that this parameter was often critical, and could vary from system to system.

All animals were treated with pulsed magnetic fields (PMF) for 12 hr./day for one month, and were then allowed to continue to regenerate for an additional month, so that their regeneration period would match that for the d.c. experiments. The space and time constraints required that these experiments take 12 months just to complete the experimental runs. Of course the limbs from

the first animals were being sectioned and analyzed before the last were placed in the fields, but data analysis and histological preparations did extend for approximately an extra two months.

The results of these experiments are summarized in Table IV. As may be seen there, the only effective frequency was 15 HZ, the same frequency which was effective in salamander limb regeneration, and which also effects repair of injured bones. Figure 5 illustrates the formation of muscle tissue which was the outstanding characteristic of these experiments. Animals treated at 15 HZ did indeed form larger amounts of blastemal tissue than did their d.c. treated counterparts. However, the outgrowths that formed tended to large and blunt, consisting mostly of apparent extensions of the preexisting structures or rather disorganized masses of tissue containing assorted islands of bone, muscle, cartilage, etc., with no discernable pattern.

Thus, these experiments satisfied part of our expectations, that of inducing the formation of larger amounts of blastemal tissue, but disappointed our hopes that larger amounts of blastema would, sui generis, lead to more complete and well-organized limb regeneration.

Reflection upon this failure led to the possible conclusion that the rat lacks a sufficiently strong "map" in its tissues to produce a well-organized regenerate from a blastema without some reinforcement or perhaps the imposition of a "map" by external means. The experiments of Stocum (3) and Bryant (4) would lead one to the conclusion that such positional information within the

FIGURE 5. ILLUSTRATION OF MUSCLE TISSUE FORMED BY PMF STIMULATION: Inset is a high-power photograph (1200 X) of the muscle fibers indicated by the arrows, which point to newly-formed muscle.

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TABLE III

PROTOCOL - PMF EXPERIMENTS

FREQUENCY	NO. ANIMALS	TREATMENT PERIOD
1 HZ	12	30 Days
2 HZ	12	30 Days
5 Hz	12	30 Days
10 HZ	12	30 Days
15 HZ	12	30 Days
25 HZ	12	30 Days
Control	24	30 Days

Total time required - 12 Months

TABLE IV

RESULTS

FREQUENCY	CARTILAGE	BONE	MUSCLE	JOINTS	OUTGROWTH
1 HZ	12	4	4	0	0.50 $\pm$ .1mm
2 HZ	10	0	0	0	0.34 $\pm$ .08mm
5 HZ	6	0	0	0	0.27 $\pm$ .12mm
10 HZ	7	1	0	0	0.11 $\pm$ .14mm
15 HZ	12	12	12	3	*1.56 $\pm$ .47mm
25 HZ	9	5	3	0	0.48 $\pm$ .23mm
CONTROL	7	0	0	0	0.18 $\pm$ .09mm

* Significantly different from Controls, p = .001

stump tissues is an absolute necessity for formation of a recognizable limb.

In addition, evidence from embryology suggests that externally applied polarizing influences are a necessary part of limb formation - external in the sense that the polarizing influence appears to be located in a region some distance from the growing tip of the limb. Thus, we felt that perhaps the reason the limbs stimulated to form tissue did not form recognizable hands was a lack of either a polarizing region (which is apparently permanently persistent in regenerating forms like salamanders, but is absent late in ontogeny in non-regenerators like chickens, frogs, people, and, apparently, rats), or lack of the positional information which lets each bit of new tissue know where it is in the limb, and thus what to become.

The d.c. experiments of section 1 seemed a natural solution to the answer of an absent polarizing influence, and the finding that a particular positioning of the electrodes was important further reinforced this conclusion. Thus, we decided to combine treatment with d.c. current and PMF-induced fields. These efforts will be outlined in section 3, below.

3. Combined Direct Current and PMF-Induced Alternating Fields:

To test the hypothesis that the principal problem faced by limbs stimulated by PMF alone is the lack of a polarizing influence, we combined PMF treatment with simultaneous d.c. current. The choice of current strength was obvious from the

experiments of section one. So was the choice of PMF from experiments in section 2. Thus, for these experiments, we implanted circuits of the type shown in section 1, made up of the battery and a 1.3 megohm resistor into the backs of 25 8-week old male Sprague-Dawley rats. The negative leads were brought down to the amputated surface of the mid-forelimb, and fixed in position as outlined section 1. The rats were then placed in cages of the type described in section 2 and treated for 12 hr. per day for 30 days with a 15 HZ pulse burst as decribed in section 2. At the end of the thirty days, the field was turned off, and the animals were allowed to regenerate for an additional 30 days. At the end of that time, they were removed from the cages, sacrificed by anesthesia and cervical dislocation, and the regenerated arms were removed for fixation and histological examination.

The results of these experiments were rather disappointing. We had naturally hoped to see some fingerlike outgrowths at least, or perhaps some indications of arrangement of the internally regenerated elements into patterns resembling carpal bone organization. What we in fact saw was a series of animals which showed almost identical regeneration to those in the section one experiments; that is, they regenerated considerable amounts of bone, cartilage, and muscle, with some joints. The organization of these elements into proximal-distal arrays was reminiscent of wrist and hand elements; but nothing remotely resembling a well-defined hand appeared. Of the 25 animals, all regenerated cartilage, 23 regenerated recognizably new bone and muscle, and joints were present in 18. The only real difference between the

animals of section one and these was in the amount of muscle. The animals in these experiments regenerated an average of 52% more than those in the section 1 experiments (as measured by stereological reconstruction of serial sections). That is of course encouraging, but leaves us far short of the desired goal of complete mammalian limb regeneration in adult animals.

The question remains as to what is wrong. Why is it that we can obtain large amounts of new tissue which can be organized into tantalizingly normal-looking groups, and yet we cannot induce truly normal limb regeneration. Several suggestions can be made which are amenable to experimental testing:

a. Something is wrong with the temporal organization of the treatment. D.C. and PMF may have to be combined in other than simultaneous fashion to be effective. Perhaps it would be best to first form a blastema with PMF, then organize it with d.c. A series of experiments with progressively delayed d.c. treatment would answer this.

b. The rat simply lacks the information in its tissues to regenerate a new limb. If one accepts the notion that the genetic material of the animal is complete, then this notion is clearly false. We simply have not learned how to elicit the necessary responses in the proper order.

c. The regeneration is somehow stopped before it can progress to an orderly completion. This may well be the best explanation at present. There is ample evidence that closure of the wound in amphibians, and the imposition of dermal tissue between the underlying tissues and the wound epithelium will stop regeneration. One of the principal adaptations of mammals to a dry land enviorment is rapid cicatrization. Under ordinary circumstances, a mammal cannot afford to leave open wounds or any length of time, both for reasons of homeostasis and protection from infection. Thus, by rapidly forming a connective tissue barrier at the wound surface, mammals may forfeit regeneration in return for rapid wound closure. To test this, one would perhaps combine electrical stimulation with treatment to prevent rapid cicatrization. The classical way to do this is with glucocorticoids, and some success with such treatments alone has been reported by Schottè (5). However, it has never been combined with electrical stimulation, and the time may be at hand to try it. In addition, there are some new compounds, notably the chitin-derived polysaccharides and glycoprotein-like substances (Chitostat, for example) which may open the way to suppression of cicatrization, and thus to complete regeneration. In view of the fact that the rat obviously can form all of the tissues necessary

to produce a new limb, it would seem obvious to try this approach.

In conclusion, it is apparent from these experiments, that complete adult limb regeneration is not currently feasible with the means employed here. However, it is equally apparent that adult mammals can form all the tissues necessary to regrow a limb, and that they can at least make some organized structures out of those tissues. It remains only for us to allow them to perfect their efforts. In addition, there is an immediate potential clinical benefit from these results. We consistently noted the formation of relatively large amounts of new muscle tissue in the PMF treated animals (at least those treated at 15 HZ). If this induced generation of de novo muscle can be controlled and reproduced at will in other species, we have at hand a potentially very useful clinical tool for the rehabilitation of personnel who have lost muscle mass through accident or combat wounds. Since the second portion of this report clearly indicates that nerve regeneration can be materially advanced by appropriate electrical stimulation, the potential for coordinated neuromuscular repair and regeneration seems particularly bright, and perhaps should be pursued with some assiduity.

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II. Studies of Electrically Stimulated Peripheral Nerve Regeneration:

The objectives of this grant were to explore the possibility that some form of bioelectrical stimulation could produce biologically improved repair of peripheral nerve injuries in a rat model. More than a decade of work with bone has solidly substantiated the hypothesis that a variety of physiologic bioelectrical stimulations, both invasive and noninvasive, can lead to the activation of dormant healing processes in fracture nonunion and result in successful healing of delayed and nonunions with restoration of function. Since limb function is so dependent upon normal neural function, and since the return of nerve function after major peripheral nerve infury in the limbs is so variable in its extent, it seemed imperative to explore further the effect of bioelectricity upon injured mammalian peripheral nerves.

A total of 3 years of research was supported under ONR grants, in collaboration with Dr. Stephen Smith and other colleagues at the University of Kentucky in Lexington. The standard laboratory rat sciatic nerve preparation was utilized, with complete transection of the sciatic nerve as the injury. Over the course of the three years, 2 different modes of electric stimulation evaluated by 2 different means of measurement were utilized, with the following results.

In the first 2 years, invasive electrical stimulation was provided by the implantation of silver-platinum electrodes designed, previously utilized in other systems, and supplied by principal investigator Stephen Smith of the University of Kentucky. Evaluation was performed utilizing integrated monophasic compound action potential measurements (IMCAP)

in vivo immediately prior to sacrifice. To summarize the experience, which was presented in detail at the 1981 meeting of the Orthopedic Research Society and published in its transactions, (1), nerves stimulated with the cathodic end of the AG-PT couple evidenced a 20% greater average area under the integrated compound action potential, suggesting a 20% improvement in nerve conduction of sensory impulses across the repaired injury site from distal to proximal.

The last year of funding was utilized to answer questions raised by certain inherent weaknesses in the model, stimulus form and evaluation mode of the previously reported work. The technique of continuous recording from wire-silastic cuff electrodes encircling the injured nerve proximally and distally was explored in order to evaluate alterations in the time course of repair. In addition, the attempt was made to explore and develop a model for noninvasive stimulation of peripheral nerve, using external electromagnectically coupled induced currents through devices supplied by Electrobiology, Inc. Finally, an attempt was made to validate the previous electrophysiologic measurements (IMCAPS) with light and electron microscopic estimations of anatomic nerve repair evidenced by regenerated myelin sheaths and axon cylinders proximal to the site of injury.

An effective wire-cuff electrode system was devised. Similarly, the technically demanding methodology of preparing the staining of peripheral nerves suitable for cross sectional myelin sheath and axon diameter measurements was established. A satisfactory model for external stimulation in animals the size of rats has not yet been worked out, due

to the difficulty of including experimental limbs and excluding control limbs in the field.

In sum, this grant has substantiated the hypothesis that bioelectric stimulation can lead to measurable (electro physiologic) improvement in peripheral nerve conduction of sensory impulses after injury and repair to rat sciatic nerve. Much additional work remains to be done in this exciting area before human trials can be commenced.

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476 – THE ROLE OF ELECTRODE POSITION IN THE ELECTRICAL INDUCTION OF LIMB REGENERATION IN SUBADULT RATS

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(Manuscript received July 2nd 1981)

SUMMARY

Eight week old rats were subjected to mid-forearm amputations, then divided into four groups. One group received no further treatment. One received inactive electrical implants, while the others received implants with either a positive or negative electrode at the dorso-ulnar surface of an unclosed amputation wound. Controls and sham-implanted animals repaired their wounds, but did not regenerate. Animals with positively charged electrodes at the surface regenerated considerable amounts of connective tissue but no distal limb structures, while those receiving negatively charged electrodes regenerated complex structures reminiscent of carpal elements. The most effective current density level employed was $1.04 \cdot 10^{-6}$ A mm^{-2}.

The results of these experiments, when compared with those performed previously, demonstrate that the position of the electrode at the wound surface is important for obtaining maximal regenerative response.

INTRODUCTION

Previous experiments in our laboratory [1–3], and in others (Borgens et al. [4,5]; Becker, [6]) have demonstrated that vertebrate limb regeneration can be markedly influenced by the application of minute electrical currents to the wounded extremity.

Most of the studies have looked at regeneration in amphibians. Only that by Becker (cited above) and by Sisken in our laboratory have focused on the stimulation of regeneration in mammals; specifically in rats. Becker used very young animals and obtained truly remarkable results in an exceptionally short time. Although many have criticized his work, no investigator has exactly repeated his experiments, and thus a critical test is lacking. Our own experience has been with older animals. And with random electrode positioning the amount of regeneration has been limited, and the quality has been poor.

In view of the previous finding [2] that dorso-ulnar placement of the stimulating electrode at the amputation surface was critical to the results in adult frogs, I decided to see if similar dorso-ulnar positioning of the electrode would produce more perfect regeneration in subadult rats. Accordingly, I have undertaken the study reported here, to ascertain whether or not the minimal results reported previously with mammals may have been due to random electrode placement.

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After the silicone had cured, the battery-resistor circuits were implanted into the animals according to the protocol in Table 1. The current values chosen (1.04×10^{-9} and 1.04×10^{-7} A) were those which had been shown to be effective in previous experiments (1.04×10^{-8} A, 1974) and a substantially lower value. In addition, sham implants were done to test for the effect of implanting the circuit and wires into the limb.

The procedure for implantation was quite simple. The animal was shaved in the dorsal midline, and the resultant patch of bare skin painted with antiseptic solution (Cidex). A 3-cm skin incision was then made from the junction of neck and thorax caudally to the thoraco-lumbar junction (see Fig. 1). A pocket was made beneath the skin and subcutaneous tissue, but external to the muscular fasciae. Then a long needle was passed up to dorso-ulnar quadrant of the forelimb in the same tissue space, so that the eye of the needle appeared in the dorsal incision. A Cidex-sterilized battery—resistor circuit with one lead clipped short and its end bared was inserted into the incision. The remaining long electrode wire (either positive or negative) the end of which was to be placed at the wound surface was threaded through the eye of the needle, which was then withdrawn down the arm, leaving the electrode wire threaded down the member. To fix the electrode at the wound surface, three simultaneous stratagems were employed. First, the proximal portion of the electrode wire was bent into a spring-like series of bends (*not* loops). Second, the electrode wire was passed through the mass of the common digital extensor muscle by means of a shorter, eyed needle. This was accomplished by folding back the skin from the wound surface to form a cuff and expose the muscle mass, by inserting the needle into the muscle just below the elbow, and by bringing it

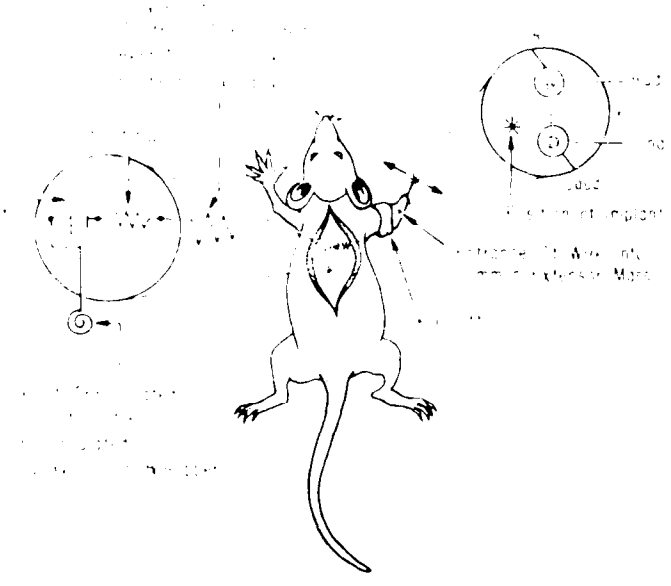

FIG. 1. Implantation procedure. See text for details.

The results indicate that careful positioning of the electrode does indeed enhance the obtained response.

EXPERIMENTAL

Materials and methods

Sixty-six one month-old Sprague-Dawley rats (half male, half female) were obtained and quartered in our laboratory animal care facilities for one month to ensure that they were healthy. As they reached 60 days of age, they were anesthetized with Nembutal and amputated across the midsection of the right antebrachium. This level was chosen because no efforts at hemostasis are required if amputation is performed at this site with a crushing instrument (scissors). None of the animals suffered significant loss of blood. This consideration was critical because I wished to leave the wounds unclosed, early experiments with amphibians having demonstrated that covering the amputation site with skin eliminates regeneration (Godlewski [7]). As may be seen from Table 1, six of the animals received no further treatment. They served as controls. The number chosen was small, because there are ample data from previous experiments to indicate that very little regeneration may be expected from such animals.

All the rest of the animals received implants of battery-resistor circuits of the sort described in our earlier experiments [2]. Briefly, the circuits consisted of a small 1.35 V mercury cell (Mallory RM 312 or equivalent), a 1/4-watt 1.3 or 13 megohm precision (1%) resistor, a pure platinum positive lead, and a multistrand teflon-insulated stainless steel negative lead (Medwire, Inc.). The circuits were assembled with silver-filled epoxy to avoid heat damage to the tiny batteries, then encapsulated first in epoxy for physical stability, and later in medical-grade silicone rubber (Dow-Corning) for immunoneutrality. Inactive circuits were made by using exhausted batteries and by not removing the insulation from the wires prior to embedding them in the epoxy. Their lack of current flow was confirmed with a picoammeter.

TABLE 1

Protocol

Animals	Sex	Condition	Current density	Electrode at surface
3	M	Control	0	0
3	F	Control	0	0
6	M	Sham	0	3+, 3−
6	F	Sham	0	3+, 3 −
12	M	Experimental	1×10^{-6} A/mm^2	6+, 6−
12	F	Experimental	1×10^{-6} A/mm^2	6+, 6−
12	M	Experimental	1×10^{-7} A/mm^2	6+, 6−
12	F	Experimental	1×10^{-7} A/mm^2	6+, 6−
Total 66				

After the silicone had
the animals according to
($1.04 \cdot 10^{-}$ and $1.04 \times$
tive in previous experim
value. In addition, sham
the circuit and wires int

The procedure for im
in the dorsal midline, ar
septic solution (Cidex).
of neck and thorax caud
pocket was made beneat
muscular fasciae. Then
the forelimb in the same
the dorsal incision. A Ci
clipped short and its en
long electrode wire (eith
placed at the wound su
was then withdrawn do
the member. To fix the
stratagems were employ
was bent into a spring-li
wire was passed through
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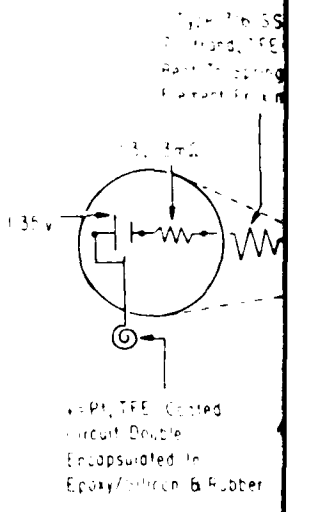

Fig. 1. Implantation proce

TABLE 2

Results

Condition	No.	Cartilage	Bone	Muscle	Joints	Outgrowth (mm)	P vs. C [a]
Control	6	6	0	0	0	0.10 ± 0.03	
Sham Op.	12	8	0	0	0	0.09 ± 0.055	N.S.
1.04×10^{-7} A/mm^2 Posit. out	12	12	1	0	0	0.46 ± 0.21	< 0.001
1.04×10^{-6} A/mm^2 posit. out	12	12	2	0	0	0.54 ± 0.21	< 0.001
1.04×10^{-7} A/mm^2 negat. out	12	12	6	5	3	1.64 ± 0.83	< 0.001
1.04×10^{-6} A/mm^2 negat. out	12	12	10	8	8	2.65 ± 1.08	< 0.001

[a] P = probability that the result is a chance occurrence; C = control.

out at the wound surface in the dorso-ulnar quadrant. The reflected skin was then returned to its normal position. Third, the electrode was caused to press gently against the exposed tissue at the end of the limb in one of two ways. In the case of the negative wire, the strands were grasped with fine forceps and pulled at 180° angles. This process peeled back the teflon insulation and generated an asterisk-shaped configuration. The pulling of the strands continued until the wire was short enough to begin to stretch the *spring* section in the back. When the strands were trimmed and released the electrode thus formed sprang back against the wound surface, and was held with sufficient force to prevent it from dislodging with the animals' movements. In the case of the positive (platinum) electrode, the single wire was stripped of its teflon insulation at the tip, then wound into a tiny flat coil which pressed against the surface of the wound in similar fashion to the asterisk of the negative electrode, using the same sort of tensioning mechanism. Both positive and negative electrodes were configured so that they covered one square millimeter, thus simplifying calculation of the gross current density from the total measured current flow in the region of the electrodes. Calculation of the boundary conditions for such electrodes would of course be exceedingly complex, and was not attempted. After placement of the electrodes was completed and their fixation tested, the dorsal incision was closed with 4-0 silk on a cuticular cutting needle. Sutures were placed very close together, so that no dressing was required, rats being very adept at chewing off attempts to dress wounds. As previously stated, no attempt was made to close the limb stump, for the reasons cited. The animals kept their wounds clean, and none developed any visible signs of infection.

Following the operative procedures, the animals were returned to their individual cages, fed and watered *ad libitum*, and maintained on a 12/12 hour light/dark cycle in quarters that were totally isolated from any other animals and ventilated with a separate filtered air supply at 23 ± 0.5°C. After 60 days they were sacrified by reanesthetization and cervical dislocation. The limbs were removed and fixed in 10% buffered formalin solution, dehydrated, embedded in paraplast, and sectioned serially at 10 μm. The sections were mounted on slides, stained with Hematoxylin/Eosin, and examined to assess the amount of regeneration which had occurred.

RESULTS

The results of these experiments can be seen in Table 2 and Fig. 2A–D. In general, there were no differences in response between males and females within the same group. Between groups, however, there were considerable differences. Each group will be treated separately below.

Control. — None of these animals exhibited more than that which is ordinarily expected after amputation — relatively rapid wound healing externally, and a minimum of differentiation internally. The skin healed over the wounded surface within a few days, and there was little outgrowth of new tissue. The response was confined to a small amount of bone removal at the tip, followed by the accumulation of rather dense fibrous tissue under the healed skin and the formation in all cases of a thin fibrocartilaginous cap over the end of the bone. Fig. 2A illustrates a typical example. Average outgrowth was 0.10 mm. The range was 0.16 to 0.08 mm.

ontrols, though the difference was not even close to significance in a *t*-test. he range was 0.01 to 0.16 mm.

Positive electrode distal. — It is evident that these animals regenerated signif antly more tissue than the controls or the sham-implanted animals. Both rrent levels produced a considerable accumulation of connective tissue under e wound surface. It averaged 0.46 mm for 1.04×10^{-7} A/mm^2, and 0.54 mm r 1.04×10^{-6} A/mm^2. The range was more extensive for the higher than the wer current (0.09 to 0.89 mm vs. 0.24 to 0.98), though the difference is ght. The difference in outgrowth was not significant at $P = 0.05$ between the o groups, but was significantly different from the controls and sham-im anted animals at the 0.001 level for both. Fig. 2B illustrates a typical case. ere is a considerable amount of connective tissue distal to the end of the ne, and a fairly extensive cartilage cap over it, but there has been no new ne, muscle, or joint formation.

Negative electrode distal. — These animals exhibited a marked degree of generation. Both groups formed outgrowths significantly ($P = 0.001$) longer an controls, sham-implanted animals, or those with the positive electrode stal. As may be seen from Table 2, the higher current level produced greater tgrowth than the lesser, and the difference is significant at the $P = 0.02$ level. e ranges were quite considerable for both groups, 0.89 to 3.42 mm for the wer level, and 0.61 to 4.03 mm for the 1.04×10^{-6} A/mm^2 level. It is in the amination of the tissue sections that the conspicuous differences between ese two groups and all the others becomes apparent. As may be seen from g. 2C, D, animals with a functioning negative electrode in the dorso-ulnar gion of the wound surface formed new bone, cartilage, muscle, and even nts. The specimen in Fig. 2D demonstrates that new structures can attain markable perfection of organization. The newly formed bone and its joint th the pre-existing proximal bony element are very nearly normal in struc re. An epiphyseal plate has formed at the distal end of the new bone, and the aline cartilage of the joint surfaces is quite typical. In addition, there is new uscle tissue formed, which has attached itself to the bone in apparently func nal relationships. Fig. 2D, a higher-power view of another specimen, illus tes that the joint formation in the regenerate parallels that usually seen in velopment. The new joint cavity forms within the cartilaginous model, and ogressively attains the structure normally seen in the adult.

In no case did I obtain anything that could be considered a proper fore w, but in many cases, the organization of the regenerating elements strongly sembled carpal structures, so that it was apparent that some attempts at nd-like organization had occurred.

SCUSSION

The results of these experiments speak fairly clearly for themselves. It is parent that stimulation of the wound surface with minute direct electrical rrent can produce a remarkable regenerative response. It is also apparent that thodic stimulation is much more effective than anodic in eliciting the sponse. The results are fundamentally similar to those we have obtained pre usly in amphibians [1,2] and rats (Sisken and Smith [3]). However, careful

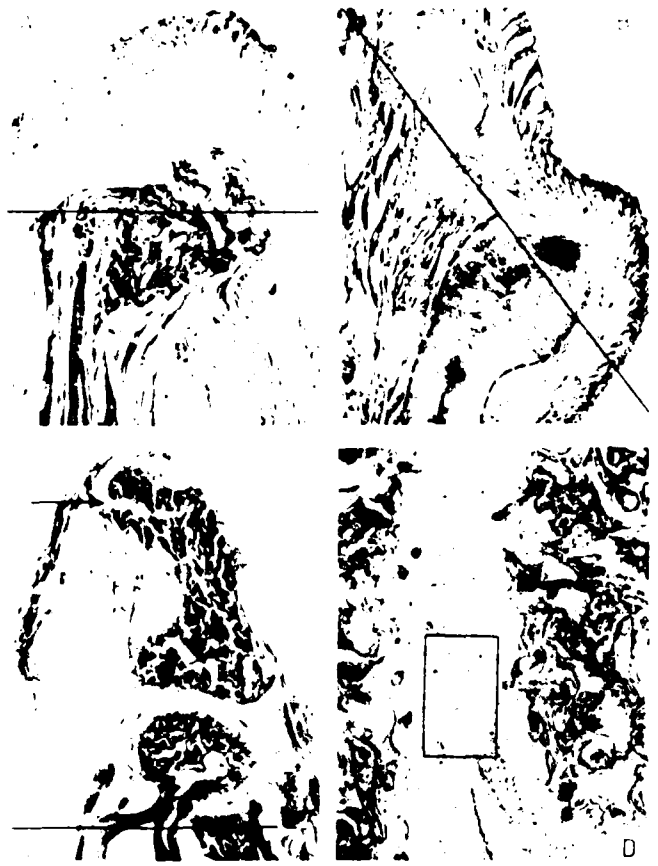

Fig. 2. A: control limb. 3.2 ×. Some bone tissue has formed at the level of amputation (traverse line), and a connective tissue/fibrocartilage cap covers the end of the limb. This response amounts to no more than wound repair, as usually seen in amputations. B: experimental limb, positive electrode distal. 3.2 ×. Considerable tissue has accumulated to the right of the transverse line, which marks the approximate level of amputation. The mass of cells extends proximally, as outlined, and presumably marks the track of the electrode wire. There is little evidence of organization in the newly formed mass. C: experimental limb, negative electrode distal. 3.2 ×. This animal gave clear evidence of extensive and well-organized regeneration. The bones and joints formed or forming are clearly reminiscent of distal limb structures. An epiphyseal plate (arrow) has formed in one of the newly formed bones. Some new muscle tissue is also evident attaching to the bone. D: experimental limb, negative electrode distal. 150 ×. This joint region from the sample specimen as that seen in C illustrates a region where joint formation is not yet complete (inset box). However, joint formation seems to have been progressing in the same way as during ontogenesis, with cells dying to form the shear plane of the joint.

Sham implanted. — As may be seen from Table 2, there was no significant difference between these animals and the controls. The healing processes and rates were identical, except that only 8 of 12 formed the fibrocartilage bone cap seen in the controls. They also grew out an average of 0.01 mm less than

controls, though the differ
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DISCUSSION

The results of these expe
apparent that stimulation o
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response. The results are fu
viously in amphibians [1,2]

attention to electrode placement in these experiments has resulted in superior results in rats, as in frogs. It seems that the stimulus is most effective when applied in the position occupied by the apical ectodermal ridge during embryogenesis. Whether there is any causal relationship is conjectural, of course, but the evidence provided by French and Bryant [8] and Stocum [9] for salamander limb regeneration strongly suggests that positional information is required for competent regeneration. Thus it may be that in attempts to induce regeneration in non-regenerators, reinforcement of rather than interference with naturally occurring polarities or organizational fields may be observed to obtain positive results. Whether such fields exist in mammals is totally unknown at present, but the results obtained here at least suggest that they may be present. If so, it may be postulated that the reason that mammals do not regenerate limbs is that their positional "maps" are too incomplete to allow for organization of competent blastemata. Thus, the reason that small properly placed *d.c.* fields are effective is that they either create "maps" (albeit imperfect ones, so far) or reinforce preexisting ones which are otherwise too weak to be effective in eliciting regenerative responses.

A second hypothesis is also worth considering. As Rose [10] has suggested, mammals may simply form scar tissue so fast that a blastema cannot form before a cicatrix does. Thus, regeneration is prevented because a competitive process "wins out". Accordingly, the incomplete results obtained in these experiments would be the result of a slight push in favor of blastema formation, but one which is not sufficient to entirely overwhelm scar formation. Regeneration is initiated, as is some organization, but cicatrization supervenes and represses the process. Rose's experiments with salt stimulation of amputated frog's limbs [11], and Schotte's experiments with scar tissue inhibition via adrenal transplants [12] argue for this hypothesis.

Yet a third hypothesis may be suggested. Our experiments with pulsed electromagnetic field stimulation of regenerating adult new limbs [13] suggest that regeneration occurs in such a way that accumulation of blastemal tissue and its differentiation can be dissociated in time. Blastemas can be induced to differentiate long before they ordinarily would, giving rise to small, incomplete limbs. It appears that regeneration is only complete if enough blastemal tissue is present to allow for the establishment of a complete pattern. If this is really so, as our evidence indicates, it may well be that mammals have a dual problem; they don't accumulate enough blastema tissue quickly enough, and their inherent positional patterns are too weak as well. To obtain perfect regeneration, perhaps it will be necessary to repress cicatrization, simultaneously stimulate the accumulation of blastemal tissue, and then reinforce or provide the blastema with the proper positional information. Such a three-step attack would perhaps involve hormonal intervention (to inhibit cicatrization), stimulation with pulsed electromagnetic fields (to obtain cell replication and a large blastema), and finally the use of a *d.c.* field (to reinforce positional information).

The experiments reported here would thus have satisfied only one of the three requirements, and one could not have expected regeneration to be complete. The use of pulsed electromagnetic fields has commenced in our laboratories, with some initially promising results in pilot experiments. However, a great deal more work will be required to distinguish whether any of the three

hypotheses, a combination of them, or an entirely new set is valid.

The role of wound closure in mammalian regeneration is unclear from these experiments. As was suggested in the materials and methods section, early evidence indicates that placing skin over a wound totally inhibits regeneration (Polezhaev [7]). Thus, we felt compelled to leave the amputation site unclosed to allow for the best possible results. The controls indicate that the simple act of leaving the wound open is insufficient to induce regeneration. However, our results with the implants seem significantly better than any obtained so far by others, so it may have been an important factor. A definitive experiment to decide the contribution of the wound closure factor probably should be undertaken in the near future.

The question also poses itself as to the mechanism of action of the electrical currents used in these experiments. There are three distinct possibilities. First, a direct effect of the electrical current on the cells themselves, which seems likely, though effects of bulk fields or charge flow cannot be entirely dismissed. The relative impedances of the cell membrane and interstitial fluids are such that virtually all of the current would be expected to pass in the extracellular compartment given the sort of continuous *d.c.* fields employed here. Second, an effect produced by electrode products; a much more likely prospect, since faradaic events no doubt do occur at the electrode surface. Spadaro and Becker [14] suggest that a likely candidate would be reduction products of molecular oxygen, such as O_2^-, H_2O_2, OH, and OH^-. They make a convincing case for such effects in the electrogenic production of bone during induced fracture healing. The voltage I employed (1.35 V) is certainly sufficient to engender their production at a type 316 stainless steel cathode. How such products would induce regeneration is unknown though a variety of mechanisms could be suggested as speculations. The third possibility is indirect stimulation by the production of fields of nonuniform ionic distribution. Cations are certainly concentrated in the vicinity of the cathode, and anions dispersed. Pilla [15] and we [16], among many others, have demonstrated that Ca^{2+} ions may have marked effects on cellular differentiation, and Jaffe [17] has postulated the role of cation currents in embryonic polarization. If the polar coordinate model of positional information advanced by Bryant [8] could be linked to a non-uniform distribution of cations, the case for the validity of both models would be considerably strengthened. However, such validation awaits very sophisticated electrochemical techniques and experimentation.

ACKNOWLEDGEMENTS

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EFFECTS OF LOW LEVELS OF DIRECT CURRENT ON PERIPHERAL NERVE REGENERATION

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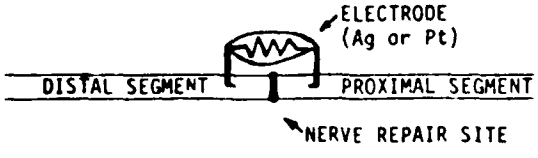
Recent work has demonstrated that very small electrical currents (1-100 na/mm^2) may strikingly influence the rate of growth and state of differentiation of adult vertebrate cells. A variety of tissues including rat bone, cartilage, muscle, and nerve have been shown to respond either altering their state of differentiation or initiating new growth and regeneration. J. Kort and C. A. L. Bassett reported at the 26th annual ORS meeting that pulsed electromagnetic fields with highly specific waveform characteristics can affect the pattern of regeneration in the transected rat sciatic nerve.

Two main theories have evolved as to the mechanism of the effect of electricity on regeneration in biological systems. A. A. Pilla has shown that pulsating current can couple to nonfaradaic electrochemical processes at cell membranes to modulate calcium ion transport. Cells exposed to induced current demonstrate a significant calcium ion efflux. Calmodulin has been suggested as a protein responsible for regulating this efflux. However, R. A. Luben has shown a greater than 90% inhibition of the response to PTH by the adenyl cyclase receptors of osteoblasts subjected to pulsed electromagnetic fields. This effect of minute electrical currents and fields upon the cyclic AMP system has also been observed by L. A. Norton et al.

The present study was undertaken to delineate the effects of low levels of galvanic current on peripheral nerve regeneration in the rat sciatic nerve. A total of fifty-six 300-325 gram male Sprague Dawley rats were used in this investigation. The sciatic nerve was exposed in each posterior thigh from the sciatic notch to the popliteal space. The nerves were transected and repaired microsurgically using 8-0 nylon suture. The right leg was implanted with an electrode according to the diagram below and the protocol in Table 1. The left sciatic nerve was simply repaired to serve as a control.



The difference in electromotive potential between silver and platinum creates a galvanic current of approximately 100 na. The interposition of a 13 megohm resistor reduces current flow to approximately 1 na.

Approximately three months following implantation, four animals underwent radioisotope evaluation by injection of their dorsal root ganglia with tritium labeled mixed amino acids. All animals underwent evaluation of their sciatic nerves physiologically by measurement of the integrated monophasic compound action potential (IMCAP) in each nerve. Following physiologic evaluation, the animals were sacrificed for histologic evaluation of the nerves.

Table 1
Protocol for Nerve Implantation

No. Rats	Implant Polarity	Current	Limb
10	+ Distal	100 na	Rt.
10	+ Distal	1 na	Rt.
10	- Distal	100 na	Rt.
10	- Distal	1 na	Rt.
6	Pt Distal	0	Rt.
6	Ag Distal	0	Rt.
56	Simple Neurorraphy	0	Lt.

The area under the curve representing the integrated monophasic compound action potential was 16% greater in sciatic nerves repaired and stimulated with 100 na of current, silver electrode distal, for an average of 12.5 weeks after repair, compared to the control contralateral repaired sciatic nerves. With the 100 na silver electrode proximal, the area was 12% less than controls. There were no significant differences in compared compound action potentials between controls and either 1 na implants or implants of either silver or platinum alone.

During the progress of this study it became apparent that the regeneration of rat sciatic nerves, even without stimulation, is virtually complete at 3 months. It is our opinion that measurement at 4 weeks after repair with and without implantation would reveal much greater differences in IMCAP areas between stimulated and unstimulated nerves; such work is in progress. Nonetheless, this study seems to indicate a clear cut stimulation of rat sciatic nerve regeneration when the silver cathode of a 100 na Ag-Pt bimetallic electrode is placed distal to the neurorraphy site, and a comparable reduction in regeneration when the polarity is reversed.

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Limb Regeneration in Rats
Stimulation of Regeneration by Electrical Fields
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Pulsed Magnetic Field Stimulation

20 ABSTRACT (Continue on reverse side if necessary and identify by block number)

This report covers research into the stimulated regeneration of subadult rat limbs and peripheral nerves. Regeneration of limbs was stimulated by implants of D.C. devices delivering 10^{-6} A/mm^2, by pulsed magnetic fields delivered as short trains of asymmetric pulses repeating at 15 Hz, and by a combination of the two. D.C. fields

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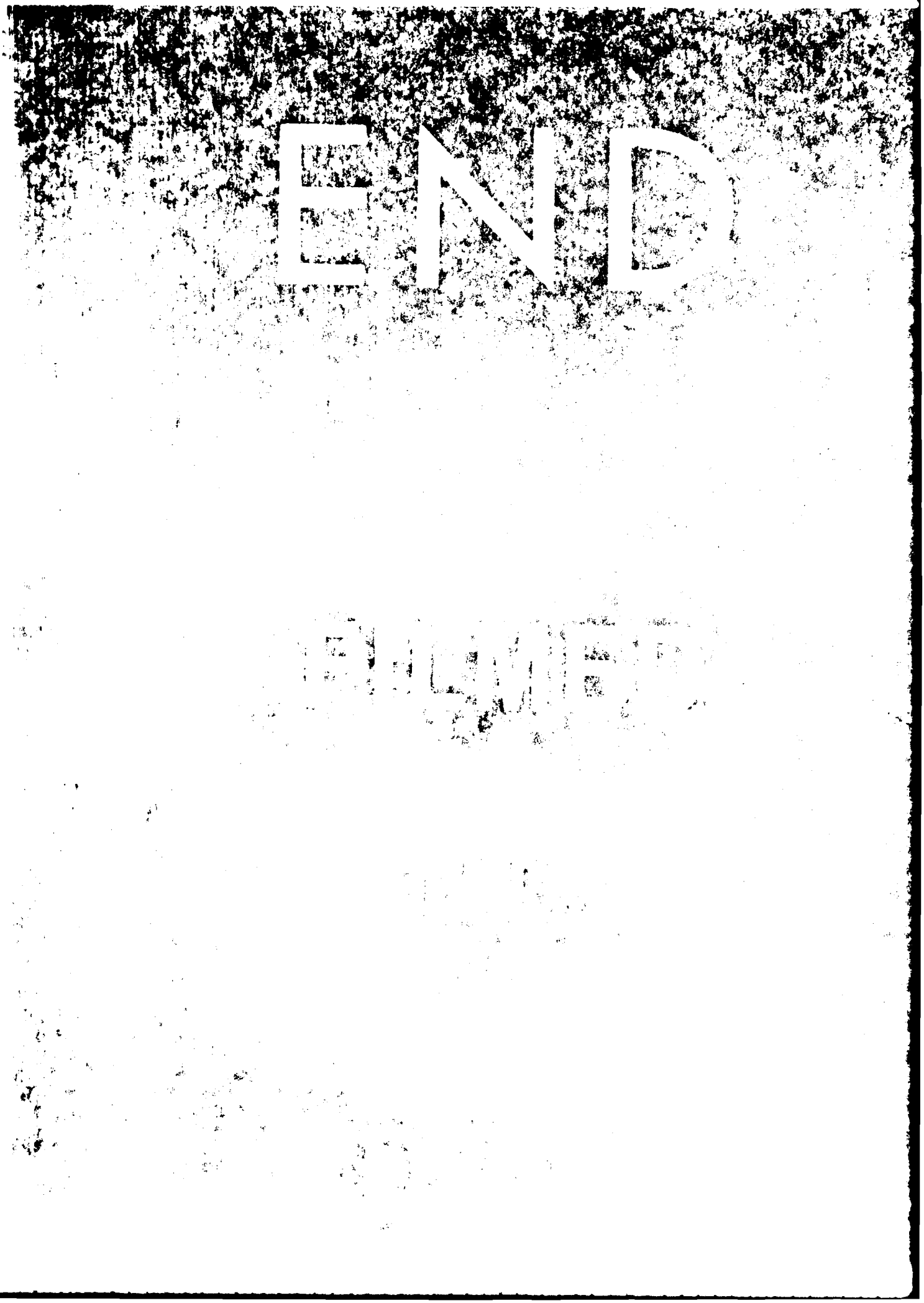
produced significant amounts of new tissue organized into proximo-distal groupings reminiscent of carpal elements. PMF produced large amounts of new tissue, chiefly muscle, but did not elicit good organization of the tissues. Combined treatment was similar to stimulation with D.C. fields, except for larger amounts of tissue. It is concluded that rats can regenerate all of the tissues necessary to recreate a lost limb, but that at present we are unable to elicit perfect limb regeneration leading to a new hand. The possible reasons for this are discussed in the full report.

Peripheral nerve regeneration was also elicited by the implantation of D.C. devices (galvanic) and by PMF. Both methods produced significantly improved nerve regeneration. Several new techniques for stimulation and for evaluation of the response have been developed, but for this report, integrated monophasic compound action potentials (IMCAPS) were the chief means of evaluation. Improvements in regeneration of the order of 20% were found.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